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Article

Comparing Breast Cancer Multiparameter Tests in the OPTIMA Prelim Trial: No Test Is More Equal Than the Others

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ABSTRACT

Background: Previous reports identifying discordance between multiparameter tests at the individual patient level have been largely attributed to methodological shortcomings of multiple in silico studies. Comparisons between tests, when performed using actual diagnostic assays, have been predicted to demonstrate high degrees of concordance. OPTIMA prelim compared predicted risk stratification and subtype classification of different multiparameter tests performed directly on the same population.

Methods: Three hundred thirteen women with early breast cancer were randomised to standard (chemotherapy and endocrine therapy) or test-directed (chemotherapy if Oncotype DX recurrence score >25) treatment. Risk stratification was also determined with, Prosigna (PAM50), MammaPrint, MammaTyper, NexCourse Breast (IHC4-AQUA) and conventional IHC4 (IHC4). Subtype classification was provided by Blueprint, MammaTyper and Prosigna.

Results: Oncotype DX predicted a higher proportion of tumours as low risk (82.1%, 95% confidence interval [CI] = 77.8% to 86.4%) than were predicted low/intermediate risk using Prosigna (65.5%, 95% CI = 60.1% to 70.9%), IHC4 (72.0%, 95% CI = 66.5 % to 77.5%), MammaPrint (61.4%, 95% CI = 55.9% to 66.9%) or NexCourse Breast (61.6%, 95% CI = 55.8% to 67.4%). Strikingly, the five tests showed only modest agreement when dichotomising results between high versus low/intermediate risk. Only 119 (39.4%) tumours were classified uniformly as either low/intermediate risk or high risk, and 183 (60.6%) were assigned to different risk categories by different tests, although 94 (31.1%) showed agreement between four or five tests. All three subtype tests assigned 59.5-62.4% of tumours to luminal A subtype, but only 121 (40.1%) were classified as luminal A by all three tests and only 58 (19.2%) were uniformly assigned as non-luminal A. Discordant subtyping was observed in 123 (40.7%) tumours.

Conclusions: Existing evidence on the comparative prognostic information provided by different tests suggests current multiparameter tests provide broadly equivalent risk information for the population of women with estrogen receptor (ER)-positive breast cancers. However, for the individual patient, tests may provide differing risk categorisation and subtype information.

INTRODUCTION

For over 40 years (1-3) the impact of tumour molecular markers on patient outcome and treatment response has been central to breast cancer management. Gene-expression profiling (4;5) to describe the intrinsic subtypes of breast cancer was followed by the independent development, in 2004, of the first multiparameter molecular diagnostic assay stratifying breast cancer patients with estrogen receptor (ER) positive disease based on risk of relapse following treatment (6). The past decade saw a rapid expansion in the number of such multiparameter molecular residual risk tests for breast cancer patients (see (7)). These herald an era of more personalised medicine because of their potential to inform rational treatment decisions on a patient-by-patient basis. The initial goal was to identify patients who, despite "favourable" clinico-pathological characteristics, have a poor outcome following conventional endocrine treatment and to advise aggressive therapy, which may reduce relapse risk. Over time, interest has also grown in the potential for multiparameter assays to predict chemo-sensitivity (8;9). These tests may also allow an estimate of the intrinsic chemotherapy sensitivity of tumours, reducing the importance of stage information. There are women who gain little from chemotherapy and women who have clinically relevant gains. There is therefore a rationale for using stratified medicine to identify patients who may safely avoid toxicities associated with chemotherapy.

The OPTIMA trial (7) is designed as a prospective test of the effectiveness of multiparameter testing in identifying the sub-group of women with breast cancer (among those who would be routinely offered adjuvant chemotherapy based on conventional criteria) whose tumours are intrinsically insensitive to chemotherapy and for whom such treatment offers only toxicity and delay in starting more effective adjuvant endocrine therapy and radiotherapy without any clinically meaningful additional benefit. A key objective of "OPTIMA prelim", the in-built feasibility phase of OPTIMA, was to evaluate the performance of alternative multiparameter tests, to aid selection of a test for

the main study that would ensure the results of such a trial be robust and broadly applicable to the patient population, both now and in the future. Critical to this decision was the ability to compare test performance at both the population and individual patient level. Existing data directly comparing individual test performance is limited. A series of studies performing statistical comparisons between tests suggest that, at a population level, four tests (IHC4, PAM50, BCI and Oncotype DX) provided broadly equivalent prognostic information on the risk of relapse up to five years post treatment (10-12). Further studies, based largely on in silico reconstruction of existing tests from publically available gene expression datasets suggest a statistically significant degree of discordance between signatures at the individual patient level (13-17). These observations are predominantly attributed to methodological differences due the in silico reconstruction of signatures (15;17). This thesis has not, to date, been robustly tested using actual test methodologies. Limited data shows that concordance between different tests in assigning patients to similar risk groups is low (10). This is consistent with the marked differences in genes measured by different tests (See Supplementary Table 1, available online) and with the relatively modest predictive value, in terms of recurrence, offered by these tests at the individual patient level. Here we report the direct patient-level comparison of multiple commercial residual risk profiles in the OPTIMA prelim study, performed to gather information on their performance.

MATERIALS AND METHODS

Recruitment and patient samples

Optimal Personalised Treatment of early breast cancer using Multiparameter Analysis preliminary study (OPTIMA prelim, ISRCTN42400492) (18) is a multicentre study that randomly assigned women aged ≥40 with ER-positive, human epidermal growth factor receptor 2 (HER2)-negative early breast cancer and either one to nine involved axillary nodes or tumour size of 30mm or

greater (if node-negative) between standard treatment (chemotherapy followed by endocrine therapy) and test-directed therapy (7). In the test-directed arm an Oncotype DX test was performed; patients with Recurrence Scores (RSs) greater than 25 ("high" risk) were assigned chemotherapy followed by endocrine therapy, those with RSs of 25 or lower ("intermediate/low" risk) received endocrine therapy alone. Chemotherapy, selected from regimens commonly used in the UK NHS, was specified at patient registration. The study was partially blinded so that neither patients nor referring centres were aware of whether chemotherapy was assigned on the basis of Oncotype DX RS or by random assignment to the standard treatment arm. Central retesting of ER and HER2 status was performed on all patients. Following confirmation of eligibility, samples were sent to Genomic Health for Oncotype DX assays to be performed with funding from the OPTIMA prelim study. No patient outcome data is available for this analysis. All patients gave written informed consent to participate in the study. The study was approved by the South East Coast - Surrey Research Ethics Committee.

To facilitate the comparison of alternative tests a number of test vendors were approached for support (Supplementary Table 2, available online**).** Ultimately five tests in addition to Oncotype DX were included in the OPTIMA prelim study: MammaPrint/BluePrint, Prosigna (PAM50), MammaTyper, NexCourse Breast by Aqua (IHC4-AQUA) and IHC4 by conventional immunohistochemistry. Multiparameter assays were performed irrespective of patient random assignment. Vendors that did not participate expressed concerns about transposing specific tests into novel applications.

Residual tumour samples from patients were collected at a central good clinical laboratory practice pathology repository (Edinburgh UK). Tissue MicroArrays (TMAs) were constructed as previously described (19) using triplicate 0.6mm cores. TMA sections, tissue sections or extracted mRNA were provided either to the Ontario Institute for Cancer Research (Prosigna; IHC4: ER,

PgR and Ki67 by quantitative image analysis (Ariol) using standard immunohistochemistry [IHC] with HER2 testing by in situ hybridization [ISH] at UCL Advanced Diagnostics) or to Genoptix (IHC4-AQUA), Agendia (MammaPrint/Blueprint) and Stratifyer (MammaTyper). Results from individual tests were collated at the Warwick Clinical Trials Unit (CTU) for analysis.

Statistical Analysis

OPTIMA prelim was designed to recruit 300 patients to enable the kappa value for agreement between tests to be estimated with good accuracy. Assuming 70% of patients would be assigned to no chemotherapy by the test and the true kappa value was 0·8 (14), this would provide a lower 95% confidence limit of 0·73. These numbers were also sufficient to allow for the assumed proportion of patients assigned to no chemotherapy to vary from 55% to 80% (lower confidence limit for kappa varied from 0·74 to 0·72 respectively).

The proportion of tumours assigned to risk groups and/or subtypes was determined. The kappa coefficient and associated 95% (CI) was used to assess agreement between tests. The predicted benefits of endocrine therapy with or without chemotherapy individualised to patients were estimated using two nomograms, Adjuvant! (20) (version 8, without correction for HER2 status) and PREDICT (21-23). A multivariable logistic regression model using stepwise elimination was performed to determine factors predicting discordant cases. To explore the post hoc hypothesis that individual tests were more likely to agree at the extremes of their ranges, two-by-two scatterplots for the tests that provide risks scores and agreement charts for the categorisation of tumours were constructed (24). Statistical analyses were performed using the SAS statistical package (version 9.3; SAS Institute Inc., Cary, NC) and R version 3·0·3 (25). All statistical tests were two-sided and a p-value of less than 0.05 was considered statistical significant.

RESULTS

Patients

Between October 2012 and June 2014, 313 patients were randomly assigned from 35 UK hospitals (see the Notes), of whom 302 had samples available for multiparameter testing (Table 1). Eleven patients were excluded from multiparameter testing; four withdrew consent, one was ineligible and samples for six patients were insufficient for testing (Supplementary Figure 1, available online).

Results from predictive nomograms

The majority of patients recruited were either at intermediate (74.8%) or high (21.2%) risk using the Nottingham Prognostic Index (NPI) (26). All 12 patients with lower risk NPI scores ($\leq$3·4) had tumours 3·0 cm or larger in size. The median 10 year overall survival estimated by PREDICT (21-23) or Adjuvant! (20) differed by 6.2% to 8.4% reflecting expected differences between the risk estimate provided by these tools (Table 2).

Multiparameter tests

Results from all tests were available for 236 (78.1%) patients. One patient on the standard arm had insufficient invasive tumour for Oncotype DX testing, but sufficient for alternative testing. Test results were unobtainable from Prosigna for three patients; from MammaTyper for four patients; from MammaPrint for four patients and BluePrint for seven patients. IHC4 and IHC4-AQUA could not be determined for 45 (14.9%) and 31 (10.3%) patients respectively, reflecting use of TMAs for this assessment.

Risk Scores

Five tests provided quantitative or semi-quantitative risk scores and a pre-defined categorised risk assessment (low, intermediate, high). For OPTIMA prelim, Oncotype DX RS was dichotomised around 25 separating "low/intermediate" from "high" risk cases as only patients with a high risk of recurrence were allocated chemotherapy (Table 3). Using this approach for all tests (Supplementary Methods, available online), the proportion of cases classified as low/intermediate risk for Oncotype DX was 82.1% (95% CI = 77.8% to 86.4%), 72.0% (95% CI = 66.5% to 77.5%) for IHC4, 65.6% (95% CI = 60.1% to 70.9%) using Prosigna risk of recurrence score including proliferation and tumour size, 61.6% (95% CI = 55.8% to 67.4%) for IHC4-AQUA, and 61.4% (95% CI = 55.9% to 66.9%) for MammaPrint (Table 3).

Agreement between tests when patients were subdivided into combined low/intermediate versus high-risk groups using predefined cut-points was modest; Kappas ranged from 0·33 (95% CI = 0·21 to 0·44) between MammaPrint and IHC4 to 0·60 (95% CI = 0·50 to 0·70) between IHC4 and IHC4-AQUA (Table 4, Supplementary Table 3, available online). Only 119 (39.4%) tumours were uniformly classified as either low/intermediate or high by all five test; 30.8% *(n=93)* tumours were classified as low/intermediate risk by all tests, a further 8.6% (n=*26*) classified as high risk by all tests. The majority (60.6%; n=183) of tumours gave no consensus result across all five tests. However for 31.1% of tumours (n=94) agreement was observed in four of the five tests. There were also no clear differences between tests in terms of the agreement with other tests (Table 5). No statistically significant differences in clinico-pathological features between tumours that were concordant or discordant were observed (Supplementary Table 4, available online). There is no evidence from the scatterplots of risk scores that individual tests were more likely to agree at the extremes of their ranges (Supplementary Figure 2, available online). Disagreement spanning one

of three risk categories was common, e.g. low risk to intermediate risk, and disagreement spanning two categories was not infrequent, i.e. low risk to high risk (Figure 1; Supplementary Table 5, available online). An exploratory analysis using a categorisation of low versus intermediate/high risk to more closely reflect current test usage was performed (Supplementary Tables 6-7, available online) again modest agreement between tests was observed.

Intrinsic Subtypes

The three tests that provide subtype information categorised similar proportions of patients as having "luminal A" tumours (BluePrint: 60.7%, 95% CI = 55.2% to 66.3%, Prosigna: 59.5%, 95% CI = 53.9% to 65.1% and MammaTyper (combined luminal A and low-risk luminal B): 62.4%, 95% CI = 56.9% to 67.9%). Thirteen (4.3%) patients were classified as having HER2 enriched/positive tumours by at least one test. Two (0.7%) patients had basal like tumours using Prosigna™ subtyping; one of whom also had a basal like tumour using BluePrint™ but triple negative breast cancer using MammaTyper™. All these patients were classified as ER-positive and HER2-negative on central review. Agreement between all three tests providing subtype assignment was obtained for 179 (59.3%) patients; 121 (40.1%) tumours were classified as luminal A; 58 (19.2%) as all other subtypes. Discordant results across these tests were seen in 123 (40.7%) patients. Moderate agreement between tests was confirmed by Kappa statistics of 0·39 (95% CI = 0·29 to 0·50) between BluePrint and MammaTyper, 0·44 (95% CI = 0·34 to 0·54) between Prosigna and MammaTyper, and 0·55 (95% CI = 0·45-0 to 64) between BluePrint and Prosigna subtype.

Assessing relationship between the Prosigna subtyping and risk of recurrence score

Prosigna is unique amongst the multiparameter assays evaluated in providing both a subtype and a continuous risk of recurrence score (ROR) with predefined risk categories derived from an identical set of genes. All 178 tumours classified as luminal A had a ROR score below the predefined high risk cut-point, and none of the 113 luminal B tumours were classified as low-risk (Table 6). Eight tumours, all of which were centrally confirmed as ER-positive/HER2-negative were categorised into either the basal-like (n=2) or HER2-like (n=6) subtypes and these were either intermediate or high risk by ROR score respectively.

DISCUSSION

The evaluation of candidate multiparameter tests within OPTIMA prelim to determine the best assessment of risk stratification for the main OPTIMA study presented an interesting challenge given: 1) Evidence that these tests provide broadly similar prognostic information at the population level (26); 2) The use of markedly different gene panels to estimate the same endpoint; 3) The use of different technologies including immunohistochemistry, polymerase chain reaction (PCR), quantitative and semi-quantitative array-based technologies.

Previous in silico comparisons of multiple gene signatures have identified statistically significant discordance between different "diagnostic tests" (13;15-17). However, to date, this has been attributed to sub-optimal comparisons, since in the majority of studies genomic prediction scores have been estimated from published expression profiles. It has been argued that, in any direct comparison of validated diagnostic genomic assays, a high level of concordance could and should be obtained (14). In the current study we performed such a direct comparison, each commercial assay was performed as prescribed by the relevant manufacturer (although the AQUA-IHC4 assay used TMAs for convenience). What is striking is that, amongst five tests with robust independent technical and clinical validation as predictors of residual risk (MammaPrint,

Oncotype DX, Prosigna, IHC4 and IHC4-AQUA) and three that measure a recognised risk factor (molecular subtype) there is marked disagreement across *all* tests. Indeed for all tests the level of agreement was "moderate" as defined by Prat et al, reaching only level 3 reproducibility (κ0.40-0.59) (14). This suggests that agreement for risk classification between different molecular tests applied to the same patient sample is on the level of agreement for pathological assessment of tumour grade.

The observed disagreement in risk categorisation for 60.6% of tumours raises questions as to how patient management may be impacted by the choice of test used for risk stratification. Interestingly there does not seem to be better correlation between tests at the extremes of their ranges (the very low and high risk tumours in our cohort) than in the mid-range. It was less common, although not infrequent, for tumours placed into the lowest risk group by one test to be assigned into the highest risk group by another.

Each test is independently validated and adopted for prediction of risk of recurrence, so what should we do when they disagree? Paradoxically the result of this study can be viewed as either predictable or unexpected, depending on perspective. From a purely biological and technical perspective it is entirely predictable that tests which measure fundamentally different genes using different technologies give dissimilar results even when each individual assay remains technically valid. For example MammaPrint and Prosigna, despite measuring the broadest range of genes (70 and 50 respectively) have only three genes in common and use different technical approaches (27;28). Even those tests measuring the same genes (IHC4, IHC4-AQUA and MammaTyper) use different technologies (PCR versus IHC) or different antibodies, detection and quantification methods.

From a clinical perspective the disagreement between multiple tests each assessing residual risk is highly perplexing. The disagreement extends to an inability to demonstrate strong agreement on molecular subtyping between tests, which again seems counter-intuitive. However, it is less surprising that disagreement between molecular subtyping, in this context predominantly between luminal A and luminal B, should exist in the absence of any clinical or molecular agreement as to the true boundary between a "luminal A" and "luminal B" cancer (16). Again, the Prosigna and BluePrint tests for subtyping have minimal gene overlap with only seven genes in common.

What about risk prediction? The prediction of disease recurrence based on clinico-pathological and molecular features of a cancer is notoriously challenging within populations and even more so at the individual patient level. Biologically and clinically aggressive cancers which, if left untreated, are destined to progress may be "cured" by surgery, radiotherapy, chemotherapy or endocrine therapy. Tests predicting risk therefore face an important challenge in that they seek to measure both the risk of recurrence based on the biology of tumours and must function within a clinical setting where biology may reflect risk that is not realised due to medical intervention. What then can we learn from comparisons between validated assays that seek to stratify patients by risk of recurrence, if indeed we can learn anything? We argue that there is value in such comparisons, even in the absence of outcome data. Each test applied in this study is externally validated and adopted or available for adoption in multiple clinical jurisdictions (6;27-32). However none is, or claims to be, the ultimate discriminator of risk for patients. This study suggests there is more than one way of predicting residual risk.

All studies have limitations. While unable to determine subtle nuances in the performance of different tests within this population, we also recognise that existing data, both from the original

studies validating individual tests and from comparisons, at a population level, of test performance in a single population (10-12) cannot provide a clear discrimination between them. No outcome data from OPTIMA prelim were available at the time of analysis. As the sample size is comparatively small it is highly unlikely that it will prove possible to compare the ability of the tests studied here to predict patient outcome.

In conclusion, in the widest and most comprehensive patient level direct diagnostic comparisons to date between multi-parametric tests of "residual risk" (after local treatment and endocrine therapy) we present further data that the proportions of patients identified as low, intermediate or high risk are broadly similar irrespective of which test is employed. However, both with respect to risk stratification and molecular sub-typing, marked differences were observed when categorisation of individual patients was considered. Such data, when considered with existing data on efficacy comparisons between different tests, support the conclusion that many current risk stratification tools are broadly equivalent and that further improvements in both prediction of relapse risk and therapeutic targeting would be of clinically significant value for patients at high risk of disease relapse (14).

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NOTES

Acknowledgments

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General Hospital, Lanarkshire, Dr Jonathan Hicks; Yeovil District Hospital, Yeovil, Dr Urmila Barthakur; York District Hospital, York, Dr Andrew Proctor.

Author contributions

John M.S. Bartlett* (Program Director & Hon. Professor, Ontario Institute of Cancer Research, Canada) was the translational research lead for the trial. He contributed to study design and managed tissue banking, the establishment of commercial relationships for undertaking multiparameter assays, the performance of laboratory assays and data analysis. He was responsible for drafting all sections of the paper and had final editorial responsibility.

Jane Bayani* (Research Scientist Ontario Institute of Cancer Research, Canada) was responsible for RNA extraction, Prosigna and IHC4 analysis and contributed to manuscript writing.

Andrea Marshall (Principal Research Fellow in Medical Statistics, University of Warwick, UK) is the trial statistician. She contributed to the statistical analysis plan, conducted the statistical analysis of the data and contributed to manuscript writing.

Janet A. Dunn (Professor of Clinical Trials, University of Warwick, UK) was the CTU lead and senior statistician for the study. She substantially contributed to the trial design, conduct including day-to-day management and monitoring as well as the statistical analysis plan.

Amy Campbell (Trial Manager, Warwick Clinical Trials Unit, University of Warwick, UK) was responsible for the day-to-day management of the trial and monitored data collection, sample collection & analysis.

Carrie Cunningham (Edinburgh Cancer Research Centre, University of Edinburgh, UK) was responsible for all aspects of sample collection, management checking pathology quality, TMA construction and sample shipping to various laboratories.

Monika S. Sobol (Edinburgh Cancer Research Centre, University of Edinburgh, UK) was responsible for all aspects of sample collection, management checking pathology quality, TMA construction and sample shipping to various laboratories.

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Christopher J. Poole (Professor of Medical Oncology, University Hospitals Coventry and Warwickshire NHS Trust, UK) contributed to the study design and its overall conduct, and advised on the clinical aspects of the trial.

David A. Cameron (Professor of Oncology & Head of Cancer Services, University of Edinburgh, UK) contributed to the study design and its overall conduct, and advised on the clinical aspects of the trial.

Helena M. Earl (Reader in Clinical Cancer Medicine, University of Cambridge Department of Oncology and NIHR Cambridge Biomedical Research Centre, UK) contributed to the study design and its overall conduct, and advised on the clinical aspects of the trial.

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Peter Canney (Consultant Oncologist, Beatson West of Scotland Cancer Centre, Glasgow, UK, retired) contributed to the study concept and design and advised on the clinical aspects of the trial.

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Christopher McCabe (Professor of Health Economics, University of Alberta, Canada) contributed to the health economics aspects of study design.

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Luke Hughes-Davies (Consultant Oncologist, Addenbrookes Hospital, Cambridge, UK) is co-chief investigator. He contributed to the concept and design of the study, its day-to-day management and overall conduct.

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Robert C. Stein (Consultant Medial Oncologist & Hon. Senior Lecturer, UCL Hospitals, London, UK) is chief investigator and lead of clinical aspects of the trial. He made substantial contributions to the concept and design of the study, its day-to-day management and overall conduct, data analysis and contributed to manuscript writing.

On behalf of the OPTIMA TMG

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Role of study sponsor

The sponsors of this study had no role in study design, data collection, analysis, interpretation, writing of the report, or the decision to publish. The authors had full access to the data and are responsible for the content of this manuscript.

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Table 1: Characteristics of the 302 patients

Characteristic	Total	
	n	%
Age years, Median(Range)	58 (40-78)	
Menopausal status of participant		
Pre/peri-menopausal	97	32.1
Postmenopausal	205	67.9
Number of involved nodes		
None	57	18.9
1-3	192	63.6
4-9	42	13.9
Positive sentinel node biopsy without clearance surgery	11	3.6
Histological grade		
1	19	6.3
2	201	66.6
3	82	27.1
Largest tumour size in mm, Median(Range)	28 (2-170)	
<=30mm	172	57.0
>30mm	130	43.0
Lymphovascular invasion reported		
No	169	56.0
Yes	122	40.4
Not Known	11	3.6
Tumour type		
Ductal	214	70.9
Lobular	65	21.5
Tubular/Cribriform	2	0.7
Mucinous	4	1.3
Micropapillary	1	0.3
Mixed	16	5.3

Table 2: Clinical risk of patients (n=302)

Risk tool	Total
Nottingham Prognostic Index, median (range)	4·6 (2·8-8·2)
≤3.4, No. (%)	12 (4.0)
>3.4 - ≤5.4, No. (%)	226 (74.8)
>5.4, No. (%)	64 (21.2)
PREDICT 10 year overall survival, median (range), %	
Endocrine therapy only	77·0 (25·1-94·6)
Chemotherapy and endocrine therapy	82·6 (39·8-95·9)
Additional benefit of chemotherapy	5·5 (1·2-25·8)
Adjuvant! 10 year risk overall survival, median (range), %	
Endocrine therapy only	68·6 (25·4-90·4)
Chemotherapy and endocrine therapy	76·4 (31·0-93·6)
Additional benefit of chemotherapy	6·8 (1·2-25·8)
Adjuvant! 10 year relapse free survival, median (range), %	
Endocrine therapy only	60·5 (22·0-82·1)
Chemotherapy and endocrine therapy	72·9 (29·1-89·4)
Additional benefit of chemotherapy	10·5 (2·7-33·3)

Table 3: Risk categorisation by each test

Risk group	Oncotype DX*	MammaPrint†	Prosigna	IHC4	IHC4-AQUA‡
No (%)	301 (99·7%)	298 (98.9%)	299 (99.0%)	257 (85.1%)	271 (89.7%)
Low risk	163 (54.2%)	183 (61.4%)	108 (36.1%)	62 (24.1%)	87 (32.1%)
Intermediate risk	84 (27.9%)	--	88 (29.4%)	123 (47.9%)	80 (29.5%)
Mid risk	--	--	--	--	55 (20.3%)
High risk	54 (17.9%)	115 (38.6%)	103 (34.5%)	72 (28.0%)	49 (18.1%)

Table 3

*Oncotype DX is divided into three risk groups with intermediate defined as Recurrence Score 18-25 for the current analysis.

†MammaPrint divides tumours into two risk groups only.

‡IHC4-AQUA divides tumours into four risk groups: low, low-mid (here called intermediate), mid and high (combined as high risk).

Table 4: Kappa statistics and 95% confidence interval (CI) for tests providing risk predictions*

Test	MammaPrint (Low), Kappa statistic (95%CI)	Prosigna (Low/ Intermediate), Kappa statistic (95%CI)	IHC4 (Low/ Intermediate), Kappa statistic (95%CI)	IHC4-AQUA† (Low/Low-Mid), Kappa statistic (95%CI)
Oncotype DX (Recurrence Score ≤25)	0·40 (0·30-0·49)	0·44 (0·33-0·54)	0·53 (0·41-0·65)	0·40 (0·30-0·51)
MammaPrint	--	0·53 (0·43-0·63)	0·33 (0·21-0·44)	0·42 (0·30-0.53)
Prosigna (Low/Intermediate)	--	--	0·39 (0·27-0·50)	0·43 (0·31-0·54)
IHC4 (Low/Intermediate)	--	--	--	0·60 (0·50-0·70)

Table 4

*Kappa statistics are for agreement between categorisation into combined low and intermediate risk versus high risk.

†IHC4-AQUA mid risk and high risk are combined for this analysis.

Table 5: Number of tests agreeing with each test

Number of other tests agreed with test	Oncotype DX, No (%)	Prosigna, No (%)	MammaPrint, No (%)	IHC4, No (%)	IHC4-AQUA, No (%)
4	119 (39.4%)	119 (39.4%)	119 (39.4%)	119 (39.4%)	119 (39.4%)
3	84 (27.8%)	77 (25.5%)	73 (24.2%)	67 (22.2%)	75 (24.8%)
2	54 (17.9%)	52 (17.2%)	47 (15.6%)	36 (11.9%)	33 (10.9%)
1	31 (10.3%)	33 (10.9%)	34 (11.2%)	25 (8.3%)	27 (9.0%)
0	13 (4.3%)	18 (6.0%)	25 (8.3%)	10 (3.3%)	17 (5.6%)
Missing	1 (0.3%)	3 (1.0%)	4 (1.3%)	45 (14.9%)	31 (10.3%)

Table 6: Relationship between Prosigna subtyping and the continuous risk of recurrence (ROR) score

Prosigna test result	Subtype			
	Luminal A No (%)	**Luminal B No (%)**	**Basal like No (%)**	**HER2 enriched No (%)**
No. of patients	178 (59.5%)	113 (37.8%)	2 (0.7%)	6 (2.0%)
Median ROR (Inter-quartile range)	37 (28-44)	70 (63-78)	53 (47-58)	76 (72-78)
Range	5-59	43-96	47-58	64-84
Risk Groups				
Low Risk	108 (60.7%)	0	0	0
Intermediate Risk	70 (39.3%)	16 (14.2%)	2 (100%)	0
High Risk	0	97 (85.8%)	0	6 (100%)

Figure 1

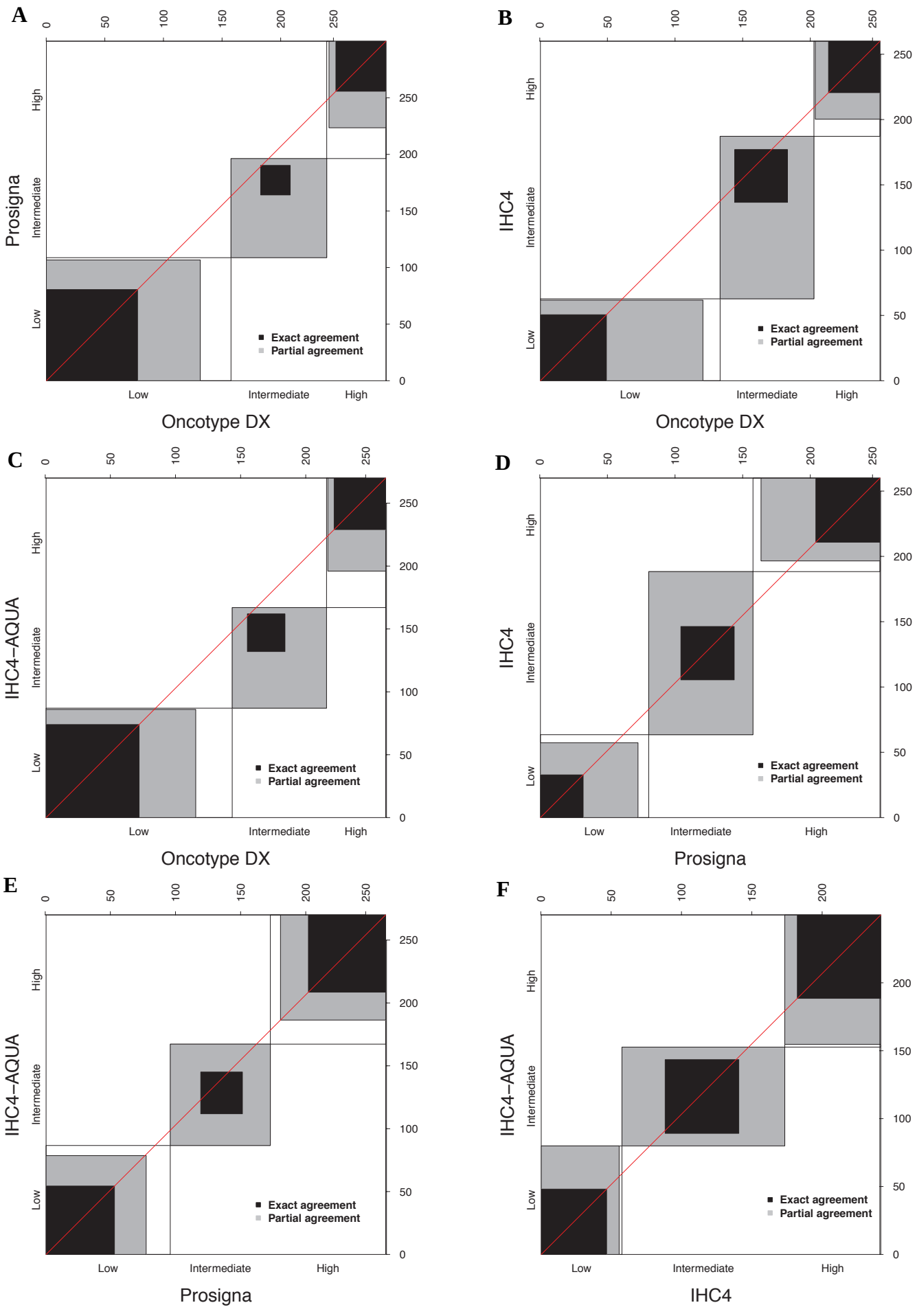

A
Prosigna
Low
Intermediate
High
Oncotype DX
Exact agreement
Partial agreement
B
IHC4
Oncotype DX
C
IHC4–AQUA
Oncotype DX
D
IHC4
Prosigna
E
IHC4–AQUA
Prosigna
F
IHC4–AQUA
IHC4
0
50
100
150
200
250

Figure Legend

Figure 1: Agreement charts for two by two comparison of tests according to risk groups. A) Prosigna against Oncotype DX; B) IHC4 against Oncotype DX; C) IHC4-AQUA against Oncotype DX; D) IHC4 against Prosigna; E) IHC4-AQUA against Prosigna; F) IHC4 against IHC4-AQUA. Only tests that provide three risk categories are included in this analysis. The Oncotype DX intermediate risk group is defined as RS 18-25. The IHC4-AQUA mid risk group was combined with the high risk group. Rectangles are drawn for each level of the test outcomes, i.e. low, intermediate and high risk, based on the row and column cumulative totals. Thus for the low risk rectangle of the test 1 vs test 2 comparison, all tumours categorised as low risk by either test are included. The boundaries of the rectangles along both axes represent the number of tumours that were categorised as that outcome for each test. Black squares within the rectangles represent exact agreement between the levels of the two tests, e.g. both low scores, and are of size based on the cell frequencies and located according to the cumulative totals of the previous levels. Grey rectangles represent partial agreement, where the scores from one test are within one level of those from the other test, i.e. a low score on one test but intermediate on the other test. White areas within the rectangle reflect disagreement by more than level, i.e. low scores on one test and high scores on the other test.

SUPPLEMENTARY MATERIALS – ONLINE ONLY

Manuscript title: Comparing breast cancer multiparameter tests in the UK OPTIMA prelim trial: All tests are equal – none are more equal than others

Supplementary Methods

For analysis, risk groups pre-defined by the test vendor were used except as described below.

Oncotype DX: Tumours are divided into three risk groups according to Recurrence Score (RS) where the intermediate risk group is defined as RS:18-31. Within OPTIMA prelim, a Recurrence Score cut-off of >25 vs. ≤25 was selected as the threshold for allocation of patients to chemotherapy or to no chemotherapy. For consistency we used the same cut-point. Tumours with an RS >25 were considered high-risk. For analyses that required two risk groups, tumours with RS ≤25 were treated as low risk. For analyses that required three risk groups, tumours with RS 18-25 were defined as intermediate risk and low risk was defined as RS <18.

Prosigna: All analyses used the risk of recurrence score including proliferation and tumour size (ROR-PT) with the vendor's predefined risk groups.

MammaPrint: The test vendor divides tumours into two groups, low and high risk and does not provide a numerical score. This test therefore could not be included in the agreement chart and scatter plot analyses.

NexCourse Breast by AQUA (IHC4-AQUA): There are four pre-defined risk groups; low risk, low-mid risk, mid risk and high risk groups. For analyses that required three risk groups, the mid risk group with a 19%-24% risk of recurrence were combined with the high risk group with a 25% risk of recurrence.

MammaTyper: This test provides subtype information but divides luminal B tumours into intermediate and high risk groups. MammaTyper classified far fewer OPTIMA prelim patients as luminal A than Prosigna and BluePrint. Therefore on the grounds of clinical applicability (plausibility) we chose to include the MammaTyper intermediate-risk luminal B group in the luminal A group.

Supplementary Table 1: Gene lists and gene overlap for the individual tests*

Prosigna		Oncotype DX	MammaPrint			IHC4	MammaTyper
ACTR3B	KRT5	BAG1*	AA555029_RC	GRHL2	RASSF7	ERBB2*	ERBB2*
				LOC100131053†			
ANLN	MAPT	BCL2*	ALDH4A1	GSTM3	RECQL5	ESR1*	ESR1*
BAG1*	MDM2	BIRC5*	AP2B1	HRASLS	RFC4	MKi67*	MKi67*
BCL2*	MELK*	CCNB1*	AYTL2	IGFBP5	RTN4RL1	PGR*	PGR*
BIRC5*	MIA	CD68	BBC3	JHDM1D	RUNDC1		
BLVRA	MKI67*	CTSL2	C16orf61 (CMC2†)	KNTC2* (NDC80*†)	SCUBE2*		
CCNB1*	MLPH	ERBB2*	C20orf46 (TMEM74B)	LETMD1	SERF1A		
CCNE1	MMP11*	ESR1*	C9orf30 (TMEFF1†)	LGP2	SLC2A3		
CDC20	MYBL2*	GRB7*	CCNE2	LIN9	SPEF1		
CDC6	MYC	GSTM1	CDC42BPA	LOC100288906	STK32B		
CDCA1 NUF2†	NAT1	MKI67*	CDCA7	LOC730018	STMN1		
CDH3	ORC6L	MMP11	CENPA	MCM6	TGFB3		
CENPF	PGR*	MYBL2	COL4A2	MELK*	TSPYL5		
CEP55	PHGDH	PGR*	DCK	MMP9	UCHL5		
CXXC5	PTTG1	SCUBE2*	DIAPH3	MS4A7	WISP1		
EGFR	RRM2	STK15	DTL	MTDH	ZNF533		
ERBB2*	SFRP1		EBF4	MYRIP			
ESR1*	SLC39A6	TFRC†	ECT2	NMU			
EXO1	TMEM45B	RPLPO†	EGLN1	NUSAP1			
FGFR4	TYMS	GUS†	ESM1	ORC6L (ORC6†)			
FOXA1	UBE2C	GAPDH†	EXT1	OXCT1			
FOXC1	UBE2T	ACTB†	FGF18	PALM2			
GPR160			FLT1	PECI			
GRB7*	MRPL19†		GMPS	PITRM1			
KIF2C	PSMC4		GNAZ	PRC1			
KNTC2* NDC80*†	SF3A1†		GPR126	QSCN6L1			
KRT14	ACTB†		GPR180	RAB6B			
KRT17	RPLP0†						

*Genes appear in more than one test.

†Genes that are used for normalisation and do not form part of the test.

Supplementary Table 2: Commercial multiparameter assays considered for inclusion in OPTIMA prelim

Test name (Designation)	Manufacturer	Included in OPTIMA prelim
Breast Cancer Index (BCI)	bioTheranostics/Qiagen, Limburg, Netherlands	No
Molecular Grade Index^{SM}	bioTheranostics/Qiagen, Limburg, Netherlands	No
Endopredict®	Sividon Diagnostics, Köln	No
Genomic Grade Index	Bordet Institute	No
MammaPrint®/ BluePrint®/ TargetPrint®	Agendia, Irvine, California	Yes
Mammastrat®	Clarient/GE Healthcare, Aliso Viejo, California	No
MammaTyper^{TM}	Stratifyer/BioNTech Diagnostics, Mainz, Germany	Yes
NexCourse® Breast by Aqua (IHC4-AQUA)	Genoptix Medical Laboratories, Carlsbad, California	Yes
Prosigna™ (PAM50)	NanoString Technologies, Seattle, Washington	Yes

Supplementary Table 3: Agreement between tests comparing low/intermediate risk versus high risk groups using pre-defined cutpoints.

	Risk Group			
	Low/Intermediate	**High**	**Total**	**%**
	Oncotype DX†			
MammaPrint				
Low	177	6	183	96.7%
High	70	44	114	38.6%
Total	247	50	297	
% concordance	71.7%	88.0%		
	Oncotype DX†			
Prosigna				
Low/Intermediate	187	8	195	95.9%
High	59	44	103	42.7%
Total	246	52	298	
% concordance	76.0%	84.6%		
	Oncotype DX†			
IHC4				
Low/Intermediate	174	11	185	94.1%
High	33	39	72	54.2%
Total	207	50	257	
% concordance	84.1%	78.0%		
	Oncotype DX†			
IHC4-AQUA				
Low/Low-Mid	161	6	167	96.4%
Mid/High	62	41	103	39.8%
Total	223	47	270	
% concordance	72.2%	87.2%		
	MammaPrint			
Prosigna				
Low/Intermediate	157	39	196	80.1%
High	25	74	99	74.7%
Total	182	113	295	
% concordance	86.3%	65.5%		
	MammaPrint			
IHC4				
Low/Intermediate	129	55	184	70.1%
High	23	47	70	67.1%
Total	152	102	254	
% concordance	84.9%	46.1%		
	MammaPrint			
IHC4-AQUA				
Low/Low-Mid	130	36	166	78.3%
Mid/High	38	65	103	63.1%
Total	168	101	269	
% concordance	77.4%	64.4%		
	Prosigna			
IHC4				
Low/Intermediate	137	47	184	74.5%
High	22	48	70	68.6%
Total	159	95	254	
% concordance	86.2%	50.5%		

	Prosigna			
IHC4-AQUA				
Low/Low-Mid	136	30	166	81.9%
Mid/High	41	61	102	59.8%
Total	177	91	268	
% concordance	76.8%	67.0%		
	IHC4			
IHC4-AQUA				
Low/Low-Mid	140	9	149	94.0%
Mid/High	35	60	95	63.2%
Total	175	69	244	
% concordance	80.0%	87.0%		

* Concordance is calculated as the percentage of patients that have been classified in the same risk group by both tests out of the row or column total respectively.

†The Oncotype DX low/intermediate risk group is defined as RS≤25.

Supplementary Table 4: Characteristics of the patients according to agreement across the five tests providing risk (Oncotype DX, Prosigna, MammaPrint, IHC4 and IHC4-AQUA)

Characteristic	Test all agree low/ interemdiate risk		Test all agree high risk		Disagree in at least one test	
	No	%	No	%	No	%
No. of patients	93	30.8	26	8.6	183	60.6
Age years, Median(Range)	58 (43-78)		57 (42-76)		57 (40-78)	
Menopausal status of participant						
Pre/peri-menopausal	30	32.3	7	26.9	60	32.8
Postmenopausal	63	67.7	19	73.1	123	67.2
Number of involved nodes						
None	18	19.3	5	19.2	34	18.6
1-3	54	58.1	15	57.7	123	67.2
4-9	16	17.2	6	23.1	20	10.9
+ve sentinel node biopsy without clearance surgery	5	5.4	0	0	6	3.3
Histological grade						
1 or 2	87	93.5	6	23.1	127	69.4
3	6	6.5	20	76.9	56	30.6
Tumour size in mm, Median(Range)	26 (7-110)		34 (8-70)		27 (2-170)	
Lymphovascular invasion reported						
No	55	59.2	9	34.6	105	57.4
Yes	35	37.6	16	61.5	71	38.8
Not Known	3	3.2	1	3.9	7	3.8

Supplementary Table 5 Agreement between pre-specified risk groups*

	Risk group				
	Low	**Intermediate**	**High**	**Total**	**% concordance**
	Oncotype DX RS†				
Prosigna					
Low	80	26	2	108	74.1%
Intermediate	55	26	6	87	29.9%
High	27	32	44	103	42.7%
Total	162	84	52	298	
% concordance	49.4%	31.0%	84.6%	kappa 0.24 (95% CI: 0.17-0.33)	
	Oncotype DX RS†				
IHC4					
Low	50	11	1	62	80.6%
Intermediate	73	40	10	123	32.5%
High	13	20	39	72	54.2%
Total	136	71	50	257	
% concordance	36.8%	56.3%	78.0%	kappa 0.27 (95% CI: 0.19-0.36)	
	Oncotype DX RS†				
IHC4-AQUA ‡					
Low	74	12	1	87	85.1%
Low-mid	45	30	5	80	37.5%
Mid/High	29	33	41	103	39.8%
Total	148	75	47	270	
% concordance	50.0%	40.0%	87.2%	kappa 0.31 (95% CI: 0.23-0.39)	
	Prosigna				
IHC4					
Low	32	24	6	62	51.6%
Intermediate	41	40	41	122	32.8%
High	8	14	48	70	68.6%
Total	81	78	95	254	
% concordance	39.5%	51.3%	50.5%	kappa 0.21 (95% CI: 0.12-0.31)	
	Prosigna				
IHC4-AQUA ‡					
Low	54	24	8	86	62.8%
Low-mid	25	33	22	80	41.3%
Mid/High	19	22	61	102	59.8%
Total	98	79	91	268	
% concordance	55.1%	41.8%	67.0%	kappa 0.33 (95% CI: 0.24-0.42)	
	IHC4				
IHC4-AQUA ‡					
Low	47	31	0	78	60.3%
Low-mid	9	53	9	71	74.6%
Mid/High	2	33	60	95	63.2%
Total	58	117	69	244	
% concordance	81.0%	45.3%	87.0%	kappa 0.49 (95% CI: 0.40-0.58)	

*Only tests that provide three risk categories are included in this analysis. Concordance is calculated as the percentage of patients that have been classified in the same risk group by both tests out of the row or column total respectively.

†The Oncotype DX low risk group is defined as RS**<18, the** intermediate risk group is defined as RS:18-25 and the high risk group as RS>25.

‡The IHC4-AQUA mid risk and high risk groups were combined.

Supplementary Table 6: Kappa statistics and 95% confidence interval (CI) for tests providing risk predictions comparing an alternative categorization of low risk versus combined intermediate and high risk

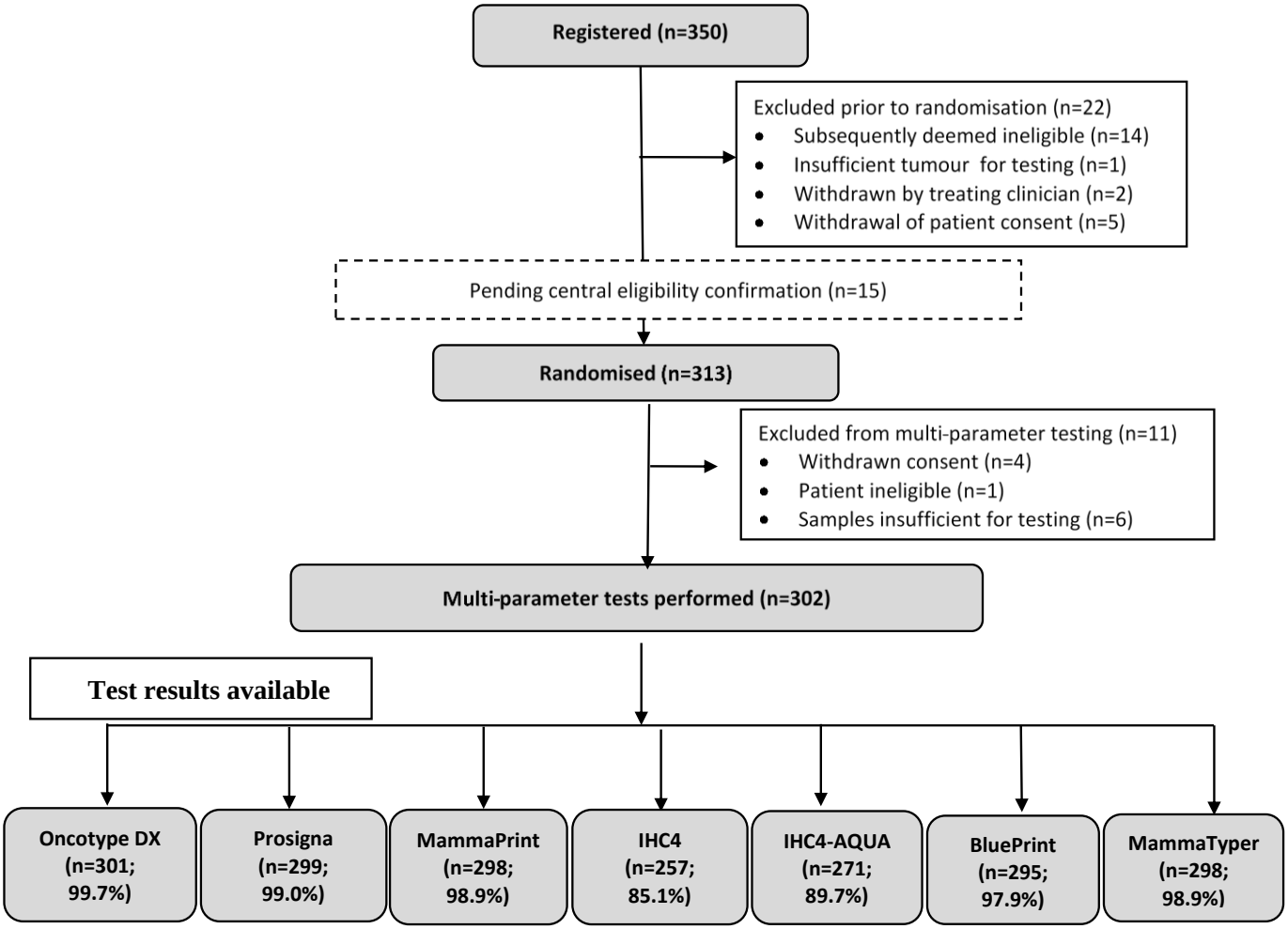
Test	**Kappa statistic (95% CI)**			
	MammaPrint (Low)	**Prosigna (Low)**	**IHC4 (Low)**	**IHC4-AQUA (Low)**
Oncotype DX (Recurrence Score <18)	0·50 (0·41-0·60)	0·28 (0·18-0·38)	0·26 (0·16-0·36)	0·38 (0·28-0·47)
MammaPrint	--	0·37 (0·28-0·46)	0·23 (0·14-0·31)	0·34 (0·25-0.43)
Prosigna (Low)	--	--	0·23 (0·11-0·36)	0·37 (0·26-0·49)
IHC4 (Low)	--	--	--	0·58 (0·46-0·69)

Supplementary Table 7: Number of tests agreeing with each test using an alternative categorization of low risk versus combined intermediate and high risk categorization*

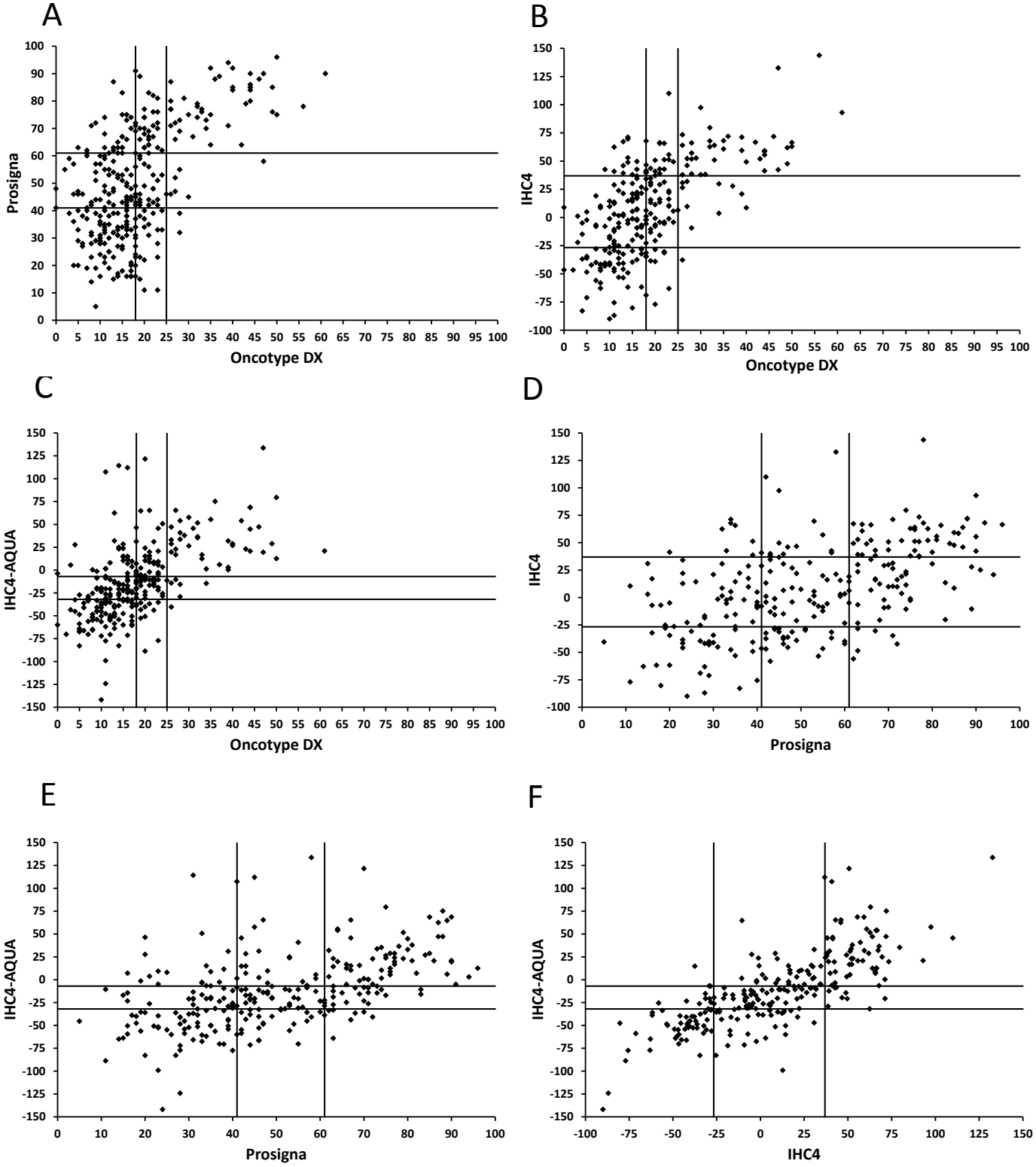
Number of other tests agreed with test	Oncotype DX, No (%)	Prosigna, No (%)	MammaPrint, No (%)	IHC4, No No (%)	IHC4-AQUA, No (%)
4	91 (30.1%)	91 (30.1%)	91 (30.1%)	91 (30.1%)	91 (30.1%)
3	68 (22.6%)	61 (20.2%)	65 (21.5%)	56 (18.6%)	51 (16.9%)
2	67 (22.2%)	81 (26.8%)	59 (19.6%)	60 (19.9%)	82 (27.1%)
1	53 (17.5%)	43 (14.3%)	63 (20.9%)	30 (9.9%)	35 (11.6%)
0	22 (7.3%)	23 (7.6%)	20 (6.6%)	20 (6.6%)	12 (4.0%)
Missing	1 (0.3%)	3 (1.0%)	4 (1.3%)	45 (14.9%)	31 (10.3%)

*Only 91 (30.1%) tumours agreed across all tests (23 [7.6%] tumours were low risk; 68 [22.5%] tumours were intermediate or high risk).

Supplementary Figure 1: Consort diagram



Supplementary Figure 2: Scatterplots of the two-by-two comparisons of tests providing risk scores



A) Prosigna against Oncotype DX; **B**) IHC4 against Oncotype DX; **C**)IHC4-AQUA against Oncotype DX; **D**) IHC4 against Prosigna; **E**) IHC4-AQUA against Prosigna; **F**) IHC4-AQUA against IHC4. Lines on the plots represent the predefined cutpoints for categorizing risk scores into low, intermediate, and high risk groups.